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# NGFI-B (Nurr77/Nr4a1) orphan nuclear receptor in rat pinealocytes: circadian expression involves an adrenergic-cyclic AMP mechanism

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**Abstract**

NGFI-B (Nur77/Nr4a1) is a member of a nuclear steroid receptor subgroup that includes the related factors Nurr1 (Nr4a2) and NOR-1 (Nr4a3). These proteins do not have recognized ligands and in fact function independently as orphan receptors with transcriptional regulatory activity. In the present study, expression of the *NGFI-B* gene in the rat pineal gland was found to exhibit a robust circadian rhythm, with elevated levels of *NGFI-B* mRNA occurring at night. The rhythm of *NGFI-B* mRNA is translated into a circadian rhythm of NGFI-B protein, which accumulates in the nucleus of pinealocytes. In addition, there is a parallel marked nocturnal increase in pineal DNA binding activity to a NGFI-B response element (NBRE, AAAGGTCA). Pharmacological studies indicate that *NGFI-B* mRNA and protein levels are elevated *via* activation of adrenergic receptors. NGFI-B protein levels are also elevated by dibutyryl cyclic AMP, as in other systems. In the pineal gland, regulation of *NGFI-B* expression also involves the AP-1 protein Fra-2, based on studies with a transgenic Fra-2 knockdown rat, in which pineal NGFI-B expression increases. This set of observations extends the number of pineal genes that are known to be regulated by Fra-2, and also provides the first indication that a member of the NGFI-B group of nuclear receptors is involved in controlling gene expression in the pineal gland.

**Keywords:** AP-1, orphan nuclear receptor.

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The rat pineal gland is a valuable model with which to study the molecular basis of gene expression because the expression of genes involved in a broad range of functions changes on a daily basis. These rhythmically expressed genes include mRNAs associated with melatonin synthesis (arylalkylamine *N*-acetyltransferase, AA-NAT; Coon *et al*. 1995), signal transduction (β-adrenergic receptor; Carter 1993; $\alpha_{1B}$-adrenergic receptor, Coon *et al*. 1997; MAPkinase phosphatase; Klein *et al*. unpublished), transcription (JunB; Carter 1994; RZRβ; Baler *et al*. 1996; inducible cyclic AMP early repressor, ICER; Maronde *et al*. 1999; Fos related antigen-2, Fra-2, Baler and Klein 1995) and thyroxine activation (type II thyroxine deiodinase; Kamiya *et al*. 1999). In all these cases, gene expression increases at night and is stimulated by norepinephrine (NE); NE release in the pineal gland is regulated by the central circadian clock located in the suprachiasmatic nucleus (SCN) The neural pathway linking the SCN to the pineal gland passes through central and peripheral structures; the gland is innervated by the *nervi conari*, a projection from the sympathetic nervous system (see Klein *et al*. 1997).

Recent microarray studies indicate that the expression of many other genes may also increase markedly at night (Humphries *et al*. 2002). This nocturnal wave of gene expression is likely to be mediated by a branching hierarchy of transcription factors, making analysis of their regulation of central importance in determining how expression of individual genes is regulated. One of the genes whose expression

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*Abbreviations used*: AA-NAT, arylalkylamine *N*-acetyltransferase; i.p., intraperitoneal; NE, norepinephrine; PBS, phosphate-buffered saline; SCN, suprachiasmatic nucleus.

  

might increase at night is the transcription factor *NGFI-B* (see Humphries *et al*. 2002). Rat *NGFI-B* was first identified in a screen for transcripts induced in neuronal cells by nerve growth factor (hence: *n*erve *g*rowth *f*actor *i*nduced gene-*B*; Milbrandt 1988). Orthologs of *NGFI-B* have been identified in other species; these are mouse *Nur77* (Hazel *et al*. 1988), human *TR3* (Nakai *et al*. 1990), and *Drosophila DHR38* (Baker *et al*. 2003). NGFI-B has been assigned the nomenclature Nr4a1, reflecting membership in the Nr4a subgroup of orphan (lacking recognized ligands) nuclear receptors; this group also includes Nur-related factor 1 (Nurr1/Nr4a2), and neuron-derived orphan receptor 1 (NOR-1/Nr4a3). Members of this subgroup exhibit homologous DNA-binding domains, and partially homologous ligand (like)-binding, and transactivation domains (see Giguere 1999).

NGFI-B was originally recognized as a homologue of the glucocorticoid receptor and was therefore of considerable functional interest as a uniquely inducible member of the nuclear steroid receptor family (Milbrandt 1988). Subsequent studies demonstrated that both NGFI-B and the related factors Nurr1 and NOR-1 are functional transcription factors in the absence of (steroid) ligand, which can bind DNA as monomers at NGFI-B response elements (NBRE, AAAGGTCA; Wilson *et al*. 1991; Giguere 1999). NGFI-B and Nurr1 can also activate transcription through DR5 elements (AGGTCA n AGGTCA; $n = 5$), acting as heterodimers with the retinoid X receptor (RXR) that binds its cognate ligand 9-*cis*-retinoic acid (Perlmann and Jansson 1995). In addition, recent studies have shown that the three members of the Nr4a group can dimerize, and activate transcription through Nur-responsive elements (NurRE, everted repeat of AAAT(G/A)(C/T)CA; Philips *et al*. 1997a; Maira *et al*. 1999). NGFI-B and related transcription factors can therefore act through a variety of genetic targets.

Functional studies of the Nr4a orphan nuclear receptors have revealed multiple roles in both developmental, and physiological regulation. Unlike NGFI-B and NOR-1 which are widely and often coexpressed, Nurr1 is relatively brain-specific (Giguere 1999), and is an essential factor for the development of dopamine neurons (Zetterstrom *et al*. 1997). Recent studies have linked Nurr1 mutations with familial Parkinson's disease (Le *et al*. 2003). In contrast, NGFI-B and NOR-1 have been primarily studied in the functional context of T lymphocyte survival, these factors acting in both a pro-apoptotic (Liu *et al*. 1994; Woronicz *et al*. 1994; Youn *et al*. 1999) and anti-apoptotic manner (Suzuki *et al*. 2003). There is also extensive evidence of a role for all three factors within the hypothalamic/pituitary/adrenal (HPA) axis (Crawford *et al*. 1995; Murphy and Conneely 1997; Philips *et al*. 1997b; Fernandez *et al*. 2000; Kovalovsky *et al*. 2002; Maira *et al*. 2003). However, the relative importance of individual factors in both the HPA, and other systems (Giguere 1999) remains to be determined, and many more functional studies are required.

In the present study we have investigated both the daily pattern of expression of *NGFI-B* mRNA, protein and DNA binding activity in the rat pineal gland, and also how expression of *NGFI-B* is regulated.

## Experimental procedures

### Animal and tissue protocols

Animal studies were conducted in accordance with both NIH guidelines on the care and use of experimental animals, and UK Home Office regulations, and local ethical review. Adult (3–4 months old) male Sprague–Dawley (CD) and DNF2 transgenic rats (Smith *et al*. 2001) rats were maintained in standard laboratory conditions either in a 14 : 10 h light–dark cycle (lights on: 05.00 h; L/D), or, for certain experiments, in constant darkness for a period of 3 days (D/D). In a pharmacological study, animals were injected with either the β-adrenoceptor agonist isoproterenol [1 mg/kg, intraperitoneal (i.p.), Sigma], or drug vehicle, at 12.00 h. Animals were killed by cervical dislocation at appropriate times of the daily cycle, and tissues were rapidly dissected. Tissue samples for northern and western analysis were snap-frozen on dry ice, and stored at −70° C prior to extraction procedures.

For immunohistochemical analysis, pineals were positioned in embedding medium (Cryo-M-Bed, Bright, Huntingdon, UK) and snap-frozen in dry ice-cooled isopentane. For *in vitro* analysis, pineals were explant cultured as described (Roseboom and Klein 1995) for 2 days prior to treatment with drug, or drug vehicle.

Unless otherwise stated, all experimental analyses were carried out in duplicate using samples derived from multiple, pooled pineal glands. Results are expressed as mean ± standard error of the mean, and statistical comparisons were conducted using Student's *t*-test.

### RNA analysis

Total cellular RNA was extracted from pairs of pooled pineal glands, fractionated on formaldehyde-agarose gels, and visualized by northern analysis as described (Humphries *et al*. 2002). Probes were labelled by random-priming (Sambrook *et al*. 1989) with [$^{32}$P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The NGFI-B probe was obtained through an RT-PCR amplification procedure as described (Humphries *et al*. 2002). Briefly, rat pineal total RNA (1 μg) was reverse transcribed using oligo dT primers (Superscript System, Life Technologies, Paisley, UK), and a 266 bp *NGFI-B* cDNA product was then amplified by PCR using a primer pair designed for the Clontech (BD Biosciences, San Jose, CA, USA) Rat 1.2 Atlas array [forward: 5′-CCGGTGACGTGCAGCAATTTTATGAC-3′ (location 1306–1332 of accession number U17254, Milbrandt, 1988), reverse: 5′-GGCTAGAATGTTGTCTATCCAGTCACC-3′ (location 1572–1545)]. The resultant PCR product was cloned into the pGEM-T vector (Promega, Madison, WI, USA) and sequence-verified. Northern blots were stripped (boiling 0.1% SDS, 3–5 × 2 min) and re-probed with a commercially available 18S cDNA (Deca-Template™, Ambion, Austin, TX, USA), and in one experiment with an *AA-NAT* cDNA probe (see Smith *et al*. 2001). Densitometric analysis of mRNA levels between samples was performed using ImageQuant™ software (3.0, Amersham Pharmacia Biotech), correcting values against the level of 18S RNA.

 

### Protein analysis

Protein extracts (cytoplasmic and nuclear) of pooled pineal glands were prepared using a modified rapid extraction procedure (Andrews and Faller 1991) in which only 75 μL of buffer A (10 mM HEPES–KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) was used in the initial phase of the extraction. Following the initial centrifugation of the crude cellular lysate, the supernatant was retained and cleared (20 800 *g*, 10 min, 4°C) for use as a cytosolic extract. Nuclear extracts were derived as described (Andrews and Faller 1991). This procedure was verified through Western analysis of the extracts with anti-synaptophysin, and anti-AA-NAT antibodies (see Results). HeLa cell nuclear extract (Geneka Biotechnology Inc., Montreal, Canada) was used as a positive control for NGFI-B detection. Western blot analysis was conducted using either a previously described procedure for the circadian samples (Carter 1994), or a modified procedure for other experiments in which proteins were resolved on 12.5% Criterion polyacrylamide gels (Bio-Rad, Hercules, CA, USA), transferred to a nitrocellulose membrane, and blocked for 2 h in 10% Blotting Grade Blocker (Bio-Rad) containing 0.2% Tween-20 and 0.05% thimerosal. Primary antisera (1 μg/mL Anti-Nur77/NGFI-B, Clone 12.14, BD Biosciences, San Jose, CA, USA; 1: 2000 Anti-Nurr-1, N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 1100 Anti-synaptophysin, clone SVP-38, Sigma, Saint Louis, MO, USA; 1 : 125 immunopurified anti-rat $AANAT_{25-200}$, number 3314) were incubated with the membranes at room temperature for 18 h. Membranes were washed, 2 × 5 min, with phosphate-buffered saline (PBS) containing 0.2% Tween-20 (TPBS), and 1 × 5 min with PBS prior to incubation with secondary antibodies: goat anti rabbit IgG-HRP-linked (1 mg per 100 mL; 3314) or goat anti-mouse IgG-HRP-linked (1 mg per 30 mL; NGFI-B/Nurr-1, or 1 mg per 40 mL synaptophysin). The membranes were washed with TPBS, 7 × 5 min, 1 × 5 min with PBS prior to protein detection by enhanced chemiluminescence using either Super Signal, Dura West (Pierce, Rockford, IL, USA) or Lumi Glo (Kirkegarrd and Perry, Gaithersburg, MD, USA) for synaptophysin and AANAT. The relative abundances of protein bands were compared using densitometric analysis (Imagequant™ 3.0, Amersham Pharmacia Biotech). The protein concentration of extracts was determined by a dye-binding method (Bradford 1976).

Immunocytochemical analysis of NGFI-B expression was performed on 8-μm sections of pineal glands prepared using a Bright OTF cryostat with Magnacut knife (Bright Instrument Company Limited, Huntingdon, UK). Sections were postfixed in 4% paraformaldehyde in PBS (5 min), permeabilized in methanol (− 20°C, 2 min) and blocked in 10% normal rabbit serum in PBS for 20 min. Sections were then incubated with an NGFI-B antiserum found to be superior for immunocytochemical analysis (M-210, 1 : 15 in PBS; Santa Cruz Biotechnology Ltd, Santa Cruz, CA, USA) for 1 h prior to washing in PBS, and incubation with a secondary antiserum (Alexa Fluor 488 goat anti-rabbit IgG; Molecular Probes Inc, Eugene, OR, USA; diluted 1 : 400 in PBS) for 30 min. Finally, sections were washed in PBS (2 × 5 min) and mounted in Vectashield plus DAPI mounting medium (Vector Laboratories, Burlingame, MA, USA). A similar protocol was used to detect pineal synaptophysin using the SVP-38 antiserum (see above, 1 : 100 in PBS) in combination with a Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA; 1 : 250 in PBS). Sections were viewed using a fluorescence microscope (Leica DM-RD), and images were captured using a Spot™ camera (1.30) and Spot Advanced Image software (Spot™ software 2.2; Diagnostic Instruments, MI, USA) before importing into Adobe Photoshop (version 4.0) for presentation.

### Protein–DNA interaction analysis

EMSA for AP-1 binding activity was performed as described previously (Carter 1994) using 0.5–1 μg of nuclear protein extract (prepared as above), and a $^{32}$P-end-labelled double-stranded oligonucleotide: 5′-TCGCCGGGCCGTGTGCGTCAGTGGCGC-3′ (which contains a NGFI-B AP-1 or 'NAP' element, − 91 to −65 of rat *NGFIB* promoter, accession number M29239; Watson and Milbrandt 1989). The EMSA analysis of NGFI-B binding activity utilized a modified protocol in which a double-stranded oligonucleotide (5′-GATCTCGAAAAGGTCACGGGA-3′) which contains a consensus NBRE element (AAAGGTCA) was $^{32}$P-labelled by filling in with the Klenow fragment of DNA polymerase (Sambrook *et al*. 1989). In additional experiments, an alternative NGFI-B binding sequence (NurRE-CON; 5′-GATCCGTGACCTTTATTC-TCAAAGGTCA-3′; see Maira *et al*. 1999) was similarly labelled. The binding reaction contained 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris/HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 5 ng of poly (dI-dC) as non-specific competitor. Protein–DNA complexes were resolved on 4% native polyacrylamide gels, and autoradiographed. The specificity of complex formation was investigated by performing the binding reactions in the presence of a molar excess of either unlabelled NAP or NBRE oligonucleotides, or alternatively unlabelled consensus sequence oligonucleotides (AP-2: GATCGAACTGACCGCCCGCGGCCCGT; SP1: ATTCGATCGGGGCGGGGCGAGC; Promega). The contribution of specific proteins to the binding complexes was investigated using antisera to either Fra-2 (2605, Smith *et al*. 2001), Jun-D (329, Santa Cruz), NGFI-B (Clone 12.14, BD) or Nurr-1 (N-20, Santa Cruz).

## Results

### Circadian and adrenergic control of NGFI-B mRNA in the rat pineal gland

Northern analysis demonstrated that the *NGFI-B* gene is expressed as a single 2.4 kb mRNA in the pineal gland (Fig. 1) as is the case in other rat tissues (Milbrandt 1988). There is a > 20-fold night/day differential in expression of *NGFI-B* mRNA, confirming the cDNA microarray analysis [Humphries *et al*. 2002; Fig. 1(a)]; summated expression data from multiple ($n = 4$ pairs) pineal glands sampled at 18.00 h (day) and 24.00 h (night) gave a 23.0 ± 3.4-fold increase in *NGFI-B* mRNA at night following correction against 18S RNA levels. In contrast to the marked night/day rhythm of *NGFI-B* expression in the rat pineal gland (Fig. 1a,b), there was no evidence of rhythmic *NGFI-B* expression in either the retina [another tissue known to exhibit circadian gene expression (Tosini and Menaker 1996)] or in a control tissue, the cerebellum (Fig. 1b).

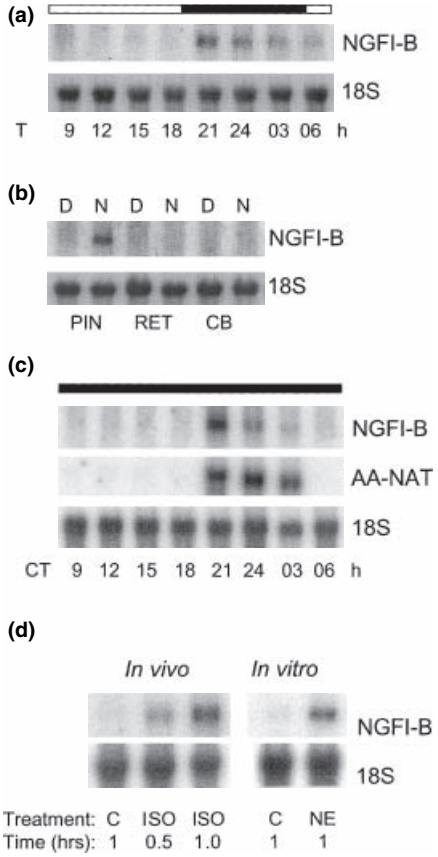


**Fig. 1** Circadian rhythm, and adrenergic regulation of NGFIB mRNA expression in the rat pineal gland. Northern blots of total pineal RNA (a,b, 6 μg/lane; c, 6 μg/lane; d, 7 μg (*in vivo*) and 4 μg (*in vitro*)/lane) hybridized sequentially with $^{32}$P-labelled-*NGFIB* and 18S cDNA probes, and additionally in (c) with an AA-NAT probe. (a) Representative analysis of pineal RNA sampled at different clock times (T) from rats maintained in a light–dark cycle (Lights off:19.00 h). Exposure times: NGFI-B, 1 day (Phosphor screen); 18S, 10 min (Phosphor screen). NGFI-B mRNA size is 2.4kb. (b) Representative analysis of pineal (PIN), retina (RET) and cerebellum (CB) RNA sampled either in the day (12.00 h, D) or night (24.00 h, N) from rats maintained in a light–dark cycle (lights off 19.00 h). Exposure times: NGFI-B, 1 day (Phosphor screen); 18S, 10 min (Phosphor screen). (c) Representative circadian time course of pineal gene expression in rats maintained in constant darkness for 3 days. Exposure times: *NGFI-B*, 2 days (Phosphor screen); AA-NAT, 12 h (Phosphor screen)18S, 15 min (Phosphor screen). Size of AA-NAT mRNA is 1.7kb. (d) Representative analyses of pineal RNA following treatment with adrenergic agonists either *in vivo*, or *in vitro*. For *in vivo* treatment, animals were injected with isoproterenol (1 mg/kg, i.p.) and killed at 0.5 and 1 h. Controls (c) were injected with vehicle and killed at 1 h. For *in vitro* treatment, explant-cultured pineal glands were treated with either norepinephrine (NE, 1 μM) or vehicle (C) for 1 h. Exposure times: *NGFI-B*, 2 days (Phosphor screen); 18S, 30 min (Phosphor screen). NE stimulated a 15.1 ± 2.2-fold increase in NGFI-B mRNA levels in explant-cultured pineal glands ($n$ = three pairs of pineal glands/group).

As indicated in the Introduction, many night/day gene rhythms in the pineal gland are truly circadian, i.e. are driven by an endogenous oscillator. Here we found this to be the case for *NGFI-B* (and *AA-NAT*); the 24-h night/day rhythm of expression was maintained in animals housed in constant darkness for 3 days (Fig. 1c), which is diagnostic for circadian regulation.

To determine if the circadian rhythm in *NGFI-B* expression is controlled through adrenergic activation as is the case for some genes in the mammalian pineal gland, we tested the effects of adrenergic agents. *In vivo* administration of isoproterenol, a β-adrenergic agonist, increased pineal *NGFI-B* mRNA levels >20-fold within 1 h of treatment (Fig. 1d). In addition, *in vitro* treatment with NE caused a 15-fold increase in NGFI-B mRNA levels (Fig. 1d).

### Rhythmic expression, and adrenergic regulation of nuclear NGFI-B protein in the rat pineal gland

Western blot analysis of pineal gland extracts (Fig. 2) demonstrated a broad NGFI-B immunoreactive protein band of predicted size (~70 kDa, Fahrner *et al*. 1990; Hazel *et al*. 1991) that was detectable during the subjective night period (CT 24 and CT 04), indicating that the circadian rhythm in NGFI-B mRNA is translated into a circadian rhythm in NGFI-B protein. A quantitative comparison of the NGFI-B protein bands at CT12 and CT24 in Fig. 2 revealed a 12-fold up-regulation of NGFI-B at CT 24. Using a similar approach with an antiserum to Nurr-1 (validated in Fig. 5c), the 65 kDa Nurr-1 protein could not be clearly detected in rat pineal gland (data not shown).

In organ culture (Fig. 3), it was found that levels of the 70 kDa NGFI-B protein band were markedly elevated in glands treated with NE. In the same experiment this treatment also up-regulated AA-NAT protein levels. NE is known to elevate cAMP in the pineal gland, which is recognized as the second messenger regulating expression of

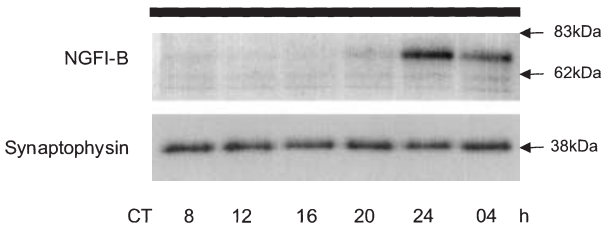


**Fig. 2** A circadian rhythm of NGFI-B protein in the rat pineal gland. Western blot analysis of nuclear (8 μg/lane) protein fractions extracted from the pineal gland of rats killed at the indicated time points of the circadian cycle (CT = circadian time). Rats were maintained in constant darkness for 3 days prior to sampling. Blots were probed with antisera against NGFI-B and synaptophysin. Arrows indicate molecular mass (kDa).

 

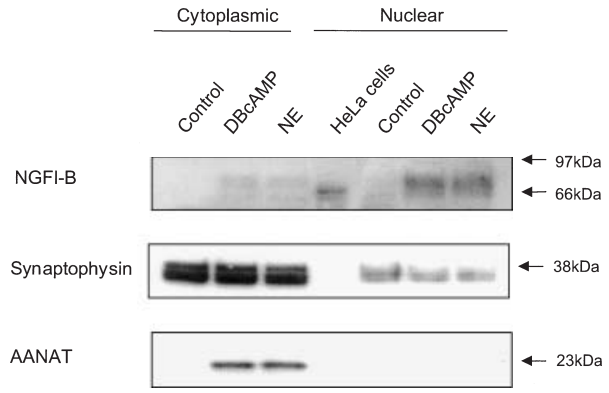


**Fig. 3** NGFI-B immunoreactive protein increases in the rat pineal gland following adrenergic and cAMP stimulation *in vitro*. Western blot analysis of cytoplasmic (50 μg/lane) and nuclear (23 μg/lane) protein fractions extracted from cultured rat pineal glands following either control treatment (control, 6 h), or treatment with dibutyryl cAMP (DBcAMP, 1 mM, 6 h) or norepinephrine (NE, 1 μM, 6 h). A commercial preparation of HeLa cell nuclear extract was also blotted. Note that the HeLa cell NGFI-B protein migrated as a discrete band at the lower molecular weight end of the broader pineal NGFI-B band (see Discussion). Blots were probed with antisera against NGFI-B, synaptophysin and AA-NAT. Arrows indicate molecular mass (kDa).

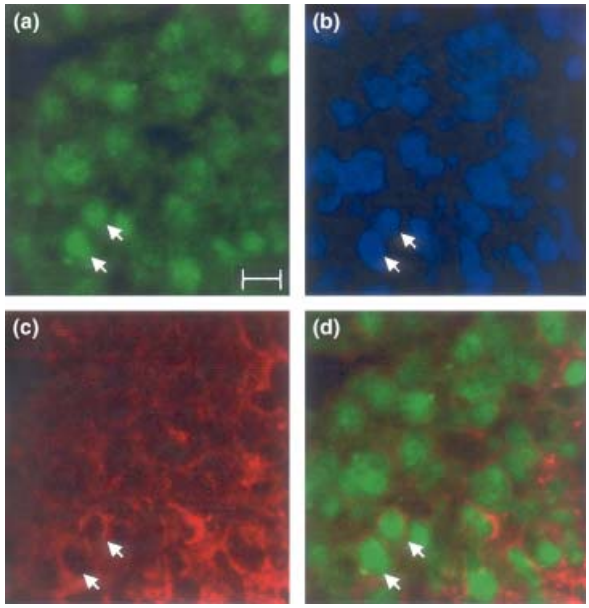


**Fig. 4** Localization of NGFI-B immunoreactive protein in the rat pineal gland. Fluorescence immunocytochemical analysis of NGFI-B protein expression in the pineal gland of rats killed at 24.00 h. A primarily nuclear localization of NGFI-B (a, green) was verified through comparison with nuclear DAPI staining (b, blue) and extra-nuclear synaptophysin (c, red). (d) is a merged image showing colocalization of NGFI-B and synaptophysin within the same cells. Scale bar = 10 μM. NGFI-B immunoreactivity was not observed when the immunocytochemical procedure was performed in the absence of primary antisera, and was markedly reduced in pineal glands sampled at 12.00 h (data not shown). Arrows are placed in each image to guide colocalization in two adjacent cells.

AA-NAT and other genes in this tissue. To examine whether expression of NGFI-B was regulated by a similar mechanism in this tissue, glands were treated with the cyclic AMP protagonist dibutyryl cAMP (Fig. 3). This resulted in an increase in the abundance of NGFI-B protein, consistent with findings in other tissues indicating the cyclic AMP regulates NGFI-B expression (Kovalovsky *et al*. 2002).

The 70 kDa NGFI-B protein band was associated with the nuclear fraction; in the same set of experiments this band comigrated with an immunoreactive band in a control HeLa cell nuclear extract (Fig. 3). Smaller, non-regulated, ~50 and 60 kDa immunoreactive bands (Figs 2 and 3) most probably represent non-specific cross-reactivity. To confirm the separation of cytoplasmic and nuclear components, these fractions were also probed for two cytoplasmic proteins, synaptophysin and AA-NAT. Both were enriched in the cytoplasmic fraction, thereby validating the fractionation method (Fig. 3; Klein *et al*. 1997).

### NGFI-B protein is expressed in the nucleus of pinealocytes

The majority of cells in the pineal gland are the melatonin-producing pinealocytes. In addition, there are other cells types, including phagocytes and interstitial cells, and vascular elements (smooth muscle and endothelial cells). To determine whether NGFI-B protein was expressed primarily in pinealocytes or other cell types, fluorescent immunohistochemistry was used. This identified NGFI-B in the nucleus of cells (Fig. 4a) and synaptophysin in the cytoplasm (Fig. 4c). Cell-by-cell analysis revealed that synaptophysin and NGFI-B are colocalized in the same cell type (Fig. 4d), and that this represented the major population of cells in the pineal gland-pinealocytes. This establishes that NGFI-B is expressed in pinealocytes. Further studies are required to examine whether NGFI-B may also be expressed in a minority population of non-pinealocyte cells in the pineal gland. The pineal gland sections presented in Fig. 4 were prepared from rats killed at 24.00 h; similar immunohistochemical analysis of pineal glands sampled at 12.00 h revealed a markedly lower abundance of NGFI-B immunoreactivity (not shown), indicating that the procedure is detecting an induced nuclear protein.

### A night/day rhythm in pineal NGFI-B DNA binding of the NGFI-B response element (NBRE)

To determine if the nocturnal increase in NGFI-B protein (Fig. 2) was associated with a rhythm in functional NGFI-B DNA binding activity, binding to a consensus NBRE was measured using EMSA. A clear night/day difference in NBRE binding activity was observed; comparison of mul-

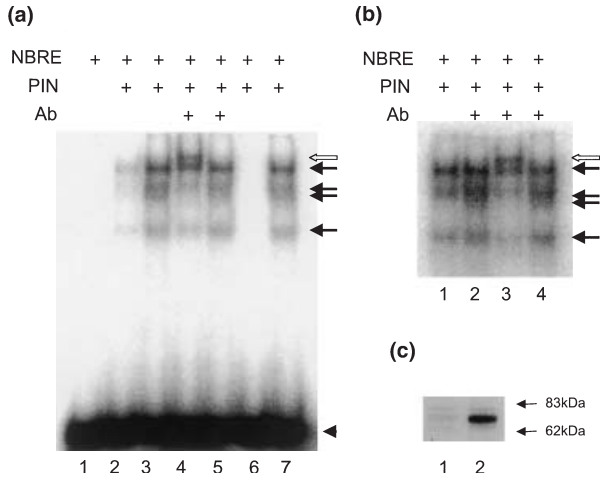


**Fig. 5** Nocturnal rhythm of NGFI-B DNA (NBRE) binding activity. (a) Representative electrophoretic mobility shift analysis (EMSA) of nuclear pineal proteins (PIN) resolved on a native polyacrylamide gel following binding with a $^{32}$P-labelled consensus response element (NBRE). Comparison of binding activity in pineal nuclear extracts at 12.00 h (lane 2) and 24.00 h (lanes 3–7). The specificity of complex formation was investigated by performing the binding reactions in the presence of a 50-fold molar excess of either unlabelled NBRE oligonucleotide (lane 6) or an unlabelled AP-2-element oligonucleotide (lane 7). An antibody (Ab) to NGFI-B was added to the binding reaction of lane 4, and a similar volume of an irrelevant antibody (anti-arginine vasopressin) to the reaction of lane 5. Note the presence of four specific binding complexes (arrows), and an abundance of free probe (arrowhead). A super-shifted band obtained in the presence of NGFI-B antibody is indicated by the open arrow. Comparison of multiple samples revealed a consistent increase in binding activity at night (2.8 ± 0.2-fold, $n = 3$ per group, $p < 0.05$, Student's *t*-test). (b) Representative EMSA similar to (a) showing a comparison of nuclear pineal protein (24.00 h) binding activity either in the absence (lane 1) or presence (lanes 2–4) of specific antisera: lane 2, Nurr-1; lane 3, NGFI-B; lane 4, anti-arginine vasopressin. Note the presence of similar complexes as in (a) (arrows), and a super-shifted band obtained in the presence of NGFI-B antibody only (open arrow). Free probe is not shown. (c) Western blot analysis of nuclear (8 μg/lane) protein fractions extracted from the adrenal gland of rats killed 1.5 h following either pentylenetetrazole treatment (Sigma, 50 mg/kg body weight, i.p., lane 2) or vehicle treatment (sterile water, 0.2 mL, i.p., lane 1). The blot was probed with an antisera against Nurr-1 protein (65 kDa). Arrows indicate molecular mass (kDa).

tiple samples revealed a consistent threefold increase in binding activity at night (Fig. 5a). The NBRE complexes were markedly reduced in the presence of an excess of unlabelled NBRE probe (Fig. 5a, lane 6). To determine whether NGFI-B was present in the NBRE binding complex, NGFI-B antiserum was added to the DNA binding reaction. This retarded migration of the NBRE complex (Fig. 5a, lane 4), consistent with the conclusion that NGFI-B is a component of this complex. In contrast, a Nurr-1 antiserum did not modify the NBRE binding complex (Fig. 5b). In additional experiments, we have also demonstrated a similar nocturnal increase in pineal protein binding to a related, NurRE-CON element (Maira *et al*. 1999; 3.3 ± 0.5-fold, $n = 3$ per group, $p < 0.05$, Student's *t*-test).

**Binding of AP-1/Fra-2 to the rat NGFI-B gene promoter**

The transcriptional mechanisms controlling *NGFI-B* expression were investigated. Previous studies have indicated that *NGFI-B* expression can be regulated by cyclic AMP, through mechanisms which do not seem to involve CRE elements; several other *cis*-acting elements have been recognized that can mediate induction of the *NGFI-B* gene (Williams and Lau 1993; Yoon and Lau 1994; Uemura *et al*. 1995; Pichon *et al*. 1996; Liu *et al*. 1999; Kovalovsky *et al*. 2002), including AP-1-like (NAP) elements (Williams and Lau 1993; Yoon and Lau 1994). Accordingly, the NAP elements present in the *NGFI-B* promoter are candidates for binding targets of Fra-2 and other AP-1 proteins, which increase at night in the pineal gland (Carter 1994; Baler and Klein 1995).

To test this, EMSA studies were performed using a fragment of the rat *NGFI-B* 5′-flanking sequence that contains the NAP element (Fig. 6). This fragment bound to a protein present in the night-time pineal gland (Fig. 6). Binding was NAP sequence-specific, as indicated by competition with unlabelled NAP oligonucleotide, but not by a

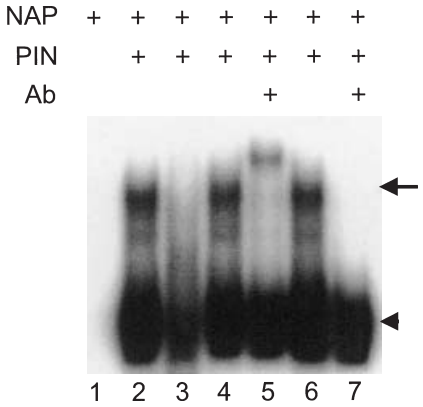


**Fig. 6** The rat NGFI-B promoter binds AP-1/Fra-2. Representative electrophoretic mobility shift analysis of nuclear pineal proteins (PIN; sampled at 23.00 h, lanes 2–7) resolved on a native polyacrylamide gel following binding with a $^{32}$P-labelled *NGFI-B* AP-1 element oligonucleotide (NAP). The specificity of complex formation was investigated by performing the binding reactions in the presence of a 20-fold molar excess of either unlabelled NAP oligonucleotide (lane 3) or an unlabelled AP-2-element oligonucleotide (lane 4). Note the presence of a specific binding complex (arrow), and an abundance of a relatively non-specific binding complex (arrowhead). Antibodies (Ab) to Fra-2 (lane 5) and Jun-D (lane 7) were added to two of the binding reactions. A preimmune serum was added to one reaction (lane 6). The non-bound probe was run off the end of the gel for enhanced DNA binding complex resolution, and is not shown.

 

similar molar excess of either AP-2 (Fig. 6) or SP-1 (not shown) consensus element oligonucleotides. The band was super-shifted by an anti Fra-2 serum (Smith *et al*. 2001), demonstrating that Fra-2 formed an important constituent of the pineal NAP-sequence binding activity (Fig. 6). Similarly, the presence of the AP-1 protein Jun-D in the complex was indicated by the finding that Jun-D antisera abrogated the NAP sequence-specific EMSA band (Fig. 6).

### NGFI-B expression is altered in a dominant negative Fra-2 transgenic rat

We examined whether changes in Fra-2 altered the expression of the *NGFI-B* gene using a line of transgenic rats that expresses a dominant negative Fra-2 molecule selectively in the pineal gland and retina; endogenous Fra-2 protein levels are reduced in these animals (Smith *et al*. 2001). Here we found that levels of *NGFI-B* mRNA at 22.00 h were significantly greater in the transgenic rats compared with wild-type animals (Fig. 7), suggesting that Fra-2 expression is negatively linked to expression of *NGFI-B*.

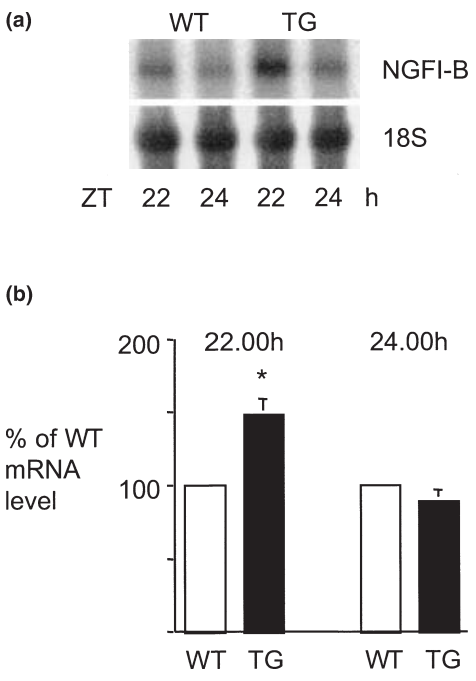


**Fig. 7** Elevated expression of nocturnal pineal NGFI-B in dominant-negative Fra-2 transgenic rats. Northern analysis of total pineal RNA (7 μg/lane) probed sequentially with $^{32}$P-labelled-*NGFIB* and 18S cDNA probes. (a) Representative analysis of pineal RNA sampled from either wild-type (WT) or DN-Fra-2 transgenic rats (TG) in the dark phase (22.00 and 24.00 h). Exposure times: *NGFI-B*, 1 day (Phosphor screen); 18S, 1 h (X-ray film). (b) Summated analysis of *NGFI-B* expression in multiple pineal glands ($n = 3$ pairs/group) sampled as in (a). Levels of *NGFI-B* mRNA were normalized against the levels of 18S RNA and are presented as a percentage of wild-type levels. *$p < 0.05$ compared with control group (Student's *t*-test).

## Discussion

Our results support the hypothesis derived from our cDNA array study (Humphries *et al*. 2002), namely that the *NGFI-B* gene is expressed in the rat pineal gland, and that expression is elevated at night. In addition, the results establish that *NGFI-B* is expressed on a circadian basis, and provide evidence that is consistent with an adrenergic mode of regulation, as is true of other pineal genes (see Klein *et al*. 1997). This is consistent with a recent microarray study (Fukuhara *et al*. 2003) that revealed high level expression of *NGFI-B* in cultured rat pineal glands, and 10-fold up-regulation by NE. The latter study also reported that *NGFI-B* is expressed at markedly higher levels than the related orphan receptors *Nurr1* and *NOR-1*, indicating that *NGFI-B* is the predominant Nr4a group member that is expressed in the rat pineal gland.

The results of the present study also show that the rhythm of *NGFI-B* mRNA is accompanied by a rhythm in levels of NGFI-B protein which is expressed at clearly detectable levels only at night, and is localized primarily within the nucleus of the pinealocyte. The night/day change in NGFI-B protein is of special importance because it indicates that mechanisms exist to facilitate rapid destruction, and that changes in the abundance of NGFI-B protein could mediate expression of other genes in the pineal gland.

NGFI-B migrated as a relatively broad protein band on sodium dodecyl sulfate–polyacrylamide gels, which has been shown to reflect the presence of differentially phosphorylated forms (Fahrner *et al*. 1990; Hazel *et al*. 1991; Tetradis *et al*. 2001). The nuclear localization of NGFI-B protein suggests it is involved in transcriptional regulation, as is the case in other systems (Wilson *et al*. 1991; Perlmann and Jansson 1995; Philips *et al*. 1997a; Giguere 1999). This is supported by the finding that NGFI-B is a component of the nocturnally elevated nuclear binding activity to a NBRE (Fig. 5a). However, the partial super-shift of this DNA binding activity in the presence of an NGFI-B antiserum suggests the presence of additional sequence-specific binding proteins which could include a contribution from other related Nr4a factors (Giguere 1999; Maira *et al*. 1999). The low relative expression of *Nurr-1* and *NOR-1* in the pineal gland compared with *NGFI-B* (see above) suggests that these factors may not be major additional components of the binding complex. This conclusion is at least partially supported by our finding that a Nurr-1 antiserum did not affect NBRE binding activity (Fig. 5c), and we were unable to detect Nurr-1 protein in western blots of pineal protein, but a NOR-1 antiserum was not available for additional studies.

Potential target genes of a transcriptional regulatory action of NGFI-B in the pineal gland are currently undefined, but would not appear to include the key enzyme in melatonin synthesis, *AA-NAT* (Coon *et al*. 1995; Klein *et al*. 1997) which is thought to be controlled via CREB (Roseboom and

Klein 1995; Maronde *et al*. 1999), and does not exhibit proximal NGFI-B response elements (Baler *et al*. 1997). A multitude of other rhythmically expressed pineal genes (Smith *et al*. 2001; Humphries *et al*. 2002; Fukuhara *et al*. 2003) is suggestive of many potential gene targets. Indeed, a recent microarray analysis of transcriptional activity in thymocytes has identified many genes that are induced by NGFI-B (Rajpal *et al*. 2003). One potential target gene in the rat pineal gland is the D4 dopamine receptor, which is rhythmically expressed in this tissue (Humphries *et al*. 2002) and has a consensus NBRE element within intron 1 [nucleotides 1848–1855 from transcription start; derived from BLAST analysis (Altschul *et al*. 1990) of rat genomic sequence AABR01060605].

In addition to defining a pineal gland *NGFI-B* rhythm, we have also discovered that expression exhibits an inverse relationship to expression of Fra-2, a major component of a nocturnal pineal AP-1 complex (Carter 1994; Baler and Klein 1995). Other recent studies using the transgenic, pineal selective, Fra-2 knockdown rat (Smith *et al*. 2001) have identified Fra-2 target genes including type II iodothyronine deiodinase and nectadrin (Smith *et al*. 2001). The demonstration of enhanced *NGFI-B* mRNA expression in the Fra-2 knockdown rat provides additional evidence that Fra-2 influences expression of several genes in the pineal gland. Our current analysis of *NGFI-B* regulation by Fra-2 has been extended beyond our previous analyses of Fra-2 function (Smith *et al*. 2001) through the identification of a promoter sequence (AP-1-like NAP element) in this target gene that binds a Fra-2-containing protein complex. Conserved NAP elements have long been recognized in the *NGFI-B* 5′ flanking sequence (Williams and Lau 1993, Yoon and Lau 1994), and have previously been shown to mediate transcriptional activation of the *NGFI-B* gene by binding Jun-D (Yoon and Lau 1994; Liu *et al*. 1999). Our demonstration that Fra-2 also associates with the NAP element is interesting because it indicates that the NAP elements (TGCGTCA), which differ from consensus AP-1 elements (TGAGTCA), are also potential mediators of the *trans*-regulatory actions of Fra-2. Unlike Jun-D, however, Fra-2 appears to exert an inhibitory action through this element (Yoon and Lau 1994; Liu *et al*. 1999). An absence of apparent Fra-2 binding to the NAP element in the study of Liu *et al*. (1999), which utilized leukaemic cell lines, is interesting because this may reflect the cell-specific expression pattern of Fra-2 (see Smith *et al*. 2001); this is most dramatically exemplified by the massive nocturnal induction of Fra-2 in the pineal (Baler and Klein 1995). Our current finding that Jun-D is also a component of pineal NAP element binding activity is consistent with previous observations (Yoon and Lau 1994; Liu *et al*. 1999), and places Jun-D and Fra-2 as candidate members of a heterodimeric complex that regulates *NGFI-B* transcription in the pineal gland through the NAP element. *In vivo* mutation analysis of the NAP element is now required to confirm a regulatory role in rhythmic pineal *NGFI-B* expression.

Previous studies of *NGFI-B* induction in other systems have collectively identified multiple pathways of regulation (Fass *et al*. 2003; Youn *et al*. 1999; Katagiri *et al*. 2000; Pekarsky *et al*. 2001; Kovalovsky *et al*. 2002; Maira *et al*. 2003). With respect to *cis*-acting mechanisms that mediate the large nocturnal increase in pineal *NGFI-B* expression, our current demonstration of regulation by cAMP implicates a CREB/cAMP response element (CRE) mode of regulation, like other pineal genes (Baler *et al*. 1997). However, CRE-like sequences are not conserved in the *NGFI-B* promoter (Uemura *et al*. 1995). The finding that cAMP regulates *NGFI-B* transcription via the MAPK pathway in corticotrophs (Kovalovsky *et al*. 2002), together with evidence that cAMP influences MAPK signalling in the pineal gland (Ho and Chik 2000), provides reason to investigate the relationship of MAPK signalling and pineal *NGFI-B* expression in the future.

In conclusion, these studies have established that the orphan nuclear receptor and transcription factor NGFI-B (Nur77/Nr4a1) is expressed in pinealocytes on a circadian basis, and is under the control of an adrenergic-cAMP mechanism. Our studies also suggest that cAMP regulates NGFI-B expression indirectly through actions involving cAMP induction of Fra-2 enriched AP-1 binding complexes and binding of these complexes to the NGFI-B promoter.

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